American Journal of Medical Genetics (Neuropsychiatric Genetics) 67:541–545 (1996)

Systematic Screening for Mutations in the Coding Region of the Human Serotonin Transporter (5-HTT) Gene Using PCR and DGGE

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Dysfunctions in serotonergic pathways may underlie several psychiatric disorders. The reuptake of serotonin (5-HT) from synaptic terminals is mediated by a specific transporter (5-HTT). Genetic variation in the gene coding for the 5-HTT protein might be involved in the predisposition to psychiatric disorders. A systematic screening of the whole coding sequence of the 5-HTT gene in mood disorder (MD) and obsessive-compulsive disorder (OCD) patients, as well as in healthy controls, using PCR and denaturing gradient gel electrophoresis (DGGE) revealed the presence of two mutations. The first was in intron 4, and the second was a C → A transversion leading to an amino-acid exchange (Leu → Met) in position 255 of the deduced protein sequence. No further occurrence of this substitution was found in an extended sample of patients and controls. Therefore, structural modifications of the 5-HTT gene do not seem to play either a major or minor role in the genetic predisposition to MD or OCD. © 1996 Wiley-Liss, Inc.

KEY WORDS: 5-HTT gene, molecular screening, PCRV, denaturing gradient gel electrophoresis (DGGE)

INTRODUCTION

Serotonin (5-HT) is a neurotransmitter that mediates a wide variety of sensory, motor, and cortical functions through multiple serotonin receptor subtypes. The primary way by which serotonergic neurons terminate neurotransmission is through the concurrent uptake of one molecule of 5-HT plus one Na^+ ion via the 5-HT transporter (5-HTT) localized in the presynaptic membrane. Recently, Lesch et al. [1993a] cloned the cDNA encoding the human brain 5-HTT. Sequence analysis identified a 630-amino-acid protein with 12 putative transmembrane domains that is 92% homologous to rat brain 5-HTT. The 5-HTT gene has been mapped to human chromosome 17q11.2 [Ramamoorthy et al., 1993] and is composed of 14 exons spanning ~35 Kb, of which ~11 Kb were sequenced, including all exons and adjacent intronic sequences [Lesch et al., 1994]. The first intron is located prior to the exon containing the translation initiation codon, and 12 introns interrupt the 5-HTT gene in the protein-coding region. The 5-HTT plays a critical role in the termination of serotonergic neurotransmission by sodium-dependent uptake of 5-HT into the presynaptic neuron, and represents an initial site of action of antidepressant drugs and neurotoxins [Amara and Kuhar, 1993; Lesch and Bengel, 1995]. Since dysfunction in serotonergic pathways may underlie several psychiatric and behavioral disorders such as mood disorders (MD), anxiety, obsessive-compulsive disorder (OCD), and substance abuse, we screened the whole coding sequence of the 5-HTT gene for mutations, using genomic DNA samples from 45 unrelated OCD patients, 56 MD patients, and 29 healthy controls.

We report on two rare novel polymorphisms in the human 5-HTT gene. They are characterized by single base-pair substitutions, namely a G → A transition in intron 4, and a C → A transversion resulting in an amino-acid exchange (Leu → Met) in position 255 of the deduced protein sequence. This coding variant was identified in a single proband (R40).

MATERIALS AND METHODS

Subjects

A sample including 45 OCD patients, 56 MD patients, and 28 healthy controls was used for preliminary screening. Demographic and clinical data are summarized in Table I. Diagnoses were assigned on the basis of the structured interview DIS-R [Robins et al.,

Received for publication January 19, 1996; revision received May 6, 1996.

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TABLE I. Demographic and Clinical Features

Diagnosis	n	Sex	Mean age ± SD (years)	Mean age-at-onset ± SD (years)	Family history
Obsessive-compulsive disorder	45	23 M, 22 F	32.9 ± 11.7	21.1 ± 10.2	33 negative 3 positive 9 unknown
Mood disorder					
Unipolar	34	7 M, 27 F	50.0 ± 14.3	36.6 ± 12.7	4 negative 13 positive 17 unknown
Bipolar	22	9 M, 13 F	46.4 ± 13.4	34.9 ± 12.2	3 negative 14 positive 5 unknown
Controls	28	10 M, 18 F	35.0 ± 10.6		

1988] according to DSM III-R [American Psychiatric Association, 1987] criteria. Healthy controls were recruited from department personnel. Only unaffected subjects with no history of DSM III-R axis 1 disorders in first-degree relatives were included in the control group. Informed consent was obtained from all probands, unrelated and of Italian descent, with antecedents from all parts of the country.

Patient R40

Patient R40 is a 56-year-old divorced woman of Northwest Italian descent, from the lower middle class. When she was 53 she was admitted to the Mood Disorders Clinic and Research Unit at San Raffaele University Hospital, Milan, and routinely assessed for axis 1 disorders. She was diagnosed as having major depressive disorder, recurrent, with mood-congruent psychotic features. Age-at-onset was 51 years. Initially she was treated with neuroleptics by a neurologist without any appreciable improvement. She was then admitted to the psychiatric ward of her city and treated with haloperidol, fluoxetine, and lorazepam.

After discharge, she experienced a 2-year period of partial remission, followed by a recurrence which made admission in our ward necessary. Clinical features were typical, including depressed mood, reduced appetite, psychomotor retardation, decreased energy, sleep disturbances, and somatic delusion. Signs of neuroleptic-induced parkinsonism were also present. On axis 2 she was diagnosed as having avoidant personality disorder. Treatment response to fluvoxamine 300 mg/day was good, and she was discharged in full remission after 2 months. At present she is no longer followed by our outpatient service. Family history was positive for two maternal uncles (mood disorder and alcohol dependence, respectively). No living relatives were available for further studies.

DNA Extraction

Genomic DNA was extracted from anticoagulated thawed blood according to the method of Lahiri and Nurnberger [1991].

Polymerase Chain Reaction (PCR)

Thirteen couples of primer were chosen to produce fragments containing the 13 coding exons (from exon 2–14) and flanks of the 5-HTT gene (Table II). Predicted sizes ranged from 126–518 bp. PCR was performed in a 25-μl volume, according to standard PCR protocols. Samples were processed in a Perkin-Elmer 480 thermal cycler (Perkin-Elmer, Monsa, Italy). After an initial denaturation at 95°C for 5 min, 35 cycles were carried out using standard temperatures for denaturation and extension (i.e., 94°C and 72°C for 1 min), and the appropriate annealing temperature for each couple of primers.

Denaturing Gradient Gel Electrophoresis (DGGE)

To detect variation in the DNA sequence, 10 μl of each PCR product were electrophoresed on parallel denaturing gradient gels, according to standard DGGE protocols [Abrams and Stanton, 1992]. The range of denaturant for each fragment was chosen using a computer-simulated melting map obtained with the MELT87 and MUTRAV programs [Lerman and Silverstein, 1987].

Sequencing of PCR Products

PCR products from heterozygous individuals were cloned into the pUC 18 *Sma*I/BAP vector (Pharmacia Biotech, Uppsala, Sweden) to confirm and characterize mutations. Plasmid DNA from single colonies were purified, and both DNA strands were cycle-sequenced (30 cycles at 95°C and 70°C for 30 sec) with primers W7-W8 and W9-W10. Nucleotide sequences were aligned and analyzed using MacVector (IBI).

Restriction Enzyme Assay

The C → A substitution abolishes a recognition site for restriction enzyme *Pvu*II (i.e., CAG/CTG). After amplification of genomic DNA using W9 and W10 primers, 5 μl of the PCR product were digested with 4U of *Pvu*II (Promega, Madison, WI) according to the manufacturer's recommendations. The digested products were separated on 12% polyacrylamide gels. The restriction profiles were visualized by ethidium bromide staining.

TABLE II. PCR Primers for Amplification of Whole Coding Sequence of 5-HTT Gene

Primer	Primer sequence	Annealing temperature	PCR product size (exon no.)
5HTT-W3	5′-CTCCTTCCTCTGTGTGTCTTTCC-3′	59°C	518 bp (2)
5HTT-W4	5′-TACTCGCAGCCTGTGATACTGAC-3′		
5HTT-W5	5′-GGGAGTGAAATTGTCTTTCATCTGCCTC-3′	66°C	188 bp (3)
5HTT-W6	5′-ACCCCACCGAGCCCTTCAGTTAC-3′		
5HTT-W7	5′-TCTCCATCTTACCCACTGCCCA-3′	57°C	277 bp (4)
5HTT-W8	5′-CCCTAACAGGCCAACCCCTACTTA-3′		
5HTT-W9	5′-TACAGAGCCTCTCAGGGGCCTTTCTTT-3′	61°C	250 bp (5)
5HTT-W10	5′-ACTGGGTTTTGAGTTTGAGAGCCTGTG-3′		
5HTT-W11	5′-CTCCCTGGAACAGCATGGTGATAAC-3′	57°C	204 bp (6)
5HTT-W12	5′-CAGGTACACATATTTCCCTCATCATCTT-3′		
5HTT-W13	5′-ATGCCCTTACCCCTGCTGTGTT-3′	57°C	180 bp (7)
5HTT-W14	5′-CTTTTACTTACGCCACTTTCAAGCT-3′		
5HTT-W15	5′-CCCTGATCTTGGAACTGTCTCAGGCG-3′	62°C	207 bp (8)
5HTT-W16	5′-AAGGGACCTGCATAGAACCCGAGGT-3′		
5HTT-W17	5′-CACCTCCTCCTCTCTCCCCTCTGTCTC-3′	65°C	177 bp (9)
5HTT-W18	5′-CTCCCACAGCCCATTTCCCCTTCCC-3′		
5HTT-W19	5′-CGTGACTGCTTGCCTGTACCTCTCA-3′	61°C	184 bp (10)
5HTT-W20	5′-TCAAAGCTGAGGGGCATGATACTCA-3′		
5HTT-W21	5′-TTGGAGGGCCCTCACCCAGGCTCT-3′	65°C	168 bp (11)
5HTT-W22	5′-GGACCCAGCTGGCTGAAGGAAGCGTC-3′		
5HTT-W23	5′-TCTTAGTCTCTGCCTCTCTTCCCCTTGGGC-3′	61°C	183 bp (12)
5HTT-W24	5′-AGTCTTTCGCCAGGGCAAGGAGGAGAAG-3′		
5HTT-W25	5′-CTCTATCTGAGTGGATATTGTTAAGGT-3′	55°C	283 bp (13)
5HTT-W26	5′-TTCTCCCAAAACAATTAGTAGTCTG-3′		
5HTT-W27	5′-ACATTGTATTTTCTTCCCAATAG-3′	55°C	126 bp (14)
5HTT-W28	5′-AGAAGCCTTTTTCCTCTCGG-3′		

RESULTS

The coding region of the 5-HTT gene from 129 unrelated subjects (including 45 with OCD, 56 with MD, and 28 healthy controls) was analyzed using DGGE. No mutations were found in 11 of the 13 PCR fragments containing the coding exons and flanks of the 5-HTT gene. However, DGGE revealed the presence of mutations in two PCR fragments. The first mutation was found in the fragment containing exon 4 and flanking regions from 2 MD patients and 1 OCD patient. Sequencing revealed the presence of one point mutation (G → A) in intron 4, 10 bp downstream from the exon-intron junction.

The second variant was identified in the coding sequence of exon 5 in 1 MD patient. This mutation is characterized by a single base-pair substitution (C → A) in nucleotide position 873 and results in an amino-acid exchange (Leu → Met) in position 255 of the deduced protein sequence (Fig. 1). At the DNA sequence level, the C → A substitution abolishes a recognition site for restriction enzyme *Pvu*II (CAG/CTG to AAG/CTT). PCR of exon 5 and flanks resulted in a 250-bp fragment. Then, depending on the presence or absence of the polymorphic *Pvu*II site, either a fragment of 152 bp (255-Met) or two fragments of 145 bp and 7 bp (255-Leu) are produced. Cleavage in a nonpolymorphic *Pvu*II site produces a constant fragment of 98 bp.

Since the C → A substitution was found in a single MD patient, we decided to increase our sample and to use the restriction assay for screening a further 67 MD patients and 74 OCD patients, as well as 76 controls for presence of the mutation (see Table III for demographic and clinical data). The substitution was not found in any other subject.

DISCUSSION

Family, twin, and adoption studies indicate genetic vulnerability to several neuropsychiatric disorders, including MD and OCD. Disturbances of serotonergic pathways have been implicated in a wide variety of neuropsychiatric disorders. Tricyclic antidepressants, such as imipramine, and the more selective 5-HT reup-

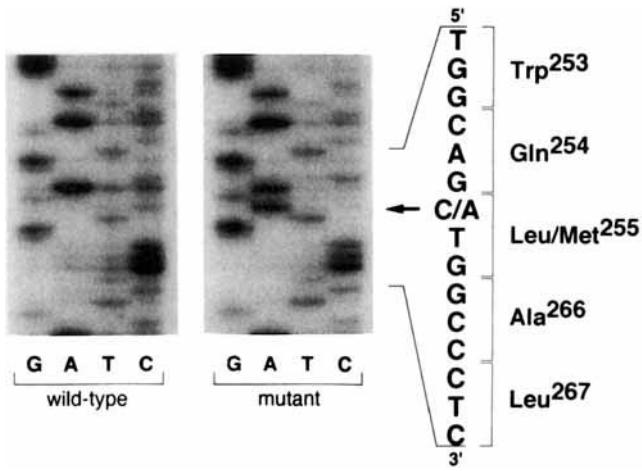

Fig. 1. DNA sequence analysis revealing point mutation found in exon 5. Arrow indicates C → A substitution in nucleotide position 873, leading to amino-acid exchange (Leu-255-Met).

TABLE III. Demographic and Clinical Features

Diagnosis	n	Sex	Mean age ± SD (years)	Mean age-at-onset ± SD (years)	Family history
Obsessive-compulsive disorder	74	38 M, 36 F	33.3 ± 12.9	19.2 ± 11.1	59 negative 15 positive
Mood disorder, bipolar	67	25 M, 42 F	44.4 ± 14.0	32.4 ± 10.7	6 negative 44 positive 17 unknown
Controls	76	27 M, 49 F	30.0 ± 8.6		

take inhibitors such as fluvoxamine and paroxetine, occupy sites overlapping the substrate-binding site and are widely used in the treatment of depression, anxiety disorders, OCD, eating disorders, and substance abuse [Marcusson and Ross, 1990]. Decreased platelet 5-HT transport [Ellis and Salmond, 1994] and reduced binding of [^{3}H]imipramine or [^{3}H]paroxetine to the brain, and platelet 5-HT uptake sites in patients with MD [Owen and Nemeroff, 1994] are among the few relatively consistent findings in psychobiological research, thus suggesting that 5-HTT may be a candidate gene in conferring susceptibility to these diseases. Decreased 5-HT uptake and inhibitor binding in MD or OCD patients may reflect a structural defect or an altered expression of 5-HTT.

To our knowledge, this is the first study reporting results from a systematic screening for nucleotide sequence variation in the human gene coding for the 5-HTT protein. However, the Leu-255-Met substitution reported here is rare and is therefore unlikely to play a role in the genetic predisposition to the diseases investigated. Furthermore, this amino-acid substitution is located in the second extracellular loop, close to the junction with the fourth transmembrane domain, and far from putative glycosylation and potential phosphorylation sites for c-AMP-dependent protein kinase or for protein kinase C [Lesch et al., 1993b].

Nevertheless, it is important to perform functional studies before ultimate conclusions are drawn.

This lack of significant polymorphisms may be explained by an intrinsic feature of the 5-HTT gene (i.e., an evolutionary conservation) or, alternatively, by the mutation-screening method. However, DGGE is routinely used to screen DNA fragments for mutations [Abrams and Stanton, 1992], and its ability to detect nearly all base changes has already allowed us to identify mutations in the dopamine D3 and D4 receptor genes [Catalano et al., 1993; Di Bella et al., 1994]. Furthermore, our findings confirm, at the genomic level and in a larger sample, previous negative results (i.e., only a silent polymorphism in a sample including 17 mood disorder patients and 4 healthy controls) from a direct cDNA sequencing study on the primary structure of 5-HTT [Lesch et al., 1995].

In conclusion, our study does not provide evidence that structural modifications of the 5-HTT gene play either a major or minor role in the genetic predisposition to OCD or MD. However, we cannot rule out that altered expression of this protein may lead to psychopathological features. In particular, sequence variations located in the 5′-flanking regulatory region of the gene (promoter and/or enhancer/silencer) could be critical to pathophysiological processes [Heils et al., 1995]. Mutational analysis of gene-regulatory sequences would provide a basis for a conclusive answer on the role of a genetic alteration in 5-HTT in psychiatric disorders.

ACKNOWLEDGMENTS

We thank Dr. M. Nöthen for his friendly advice, Dr. E. Novelli for helpful assistance, and Prof. L.S. Lerman and W. Fripp for providing the software for computational simulation.

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